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High-Efficiency Incorporation of Functional Influenza Virus Glycoproteins into Recombinant Vesicular Stomatitis Viruses

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We derived recombinant vesicular stomatitis virus (VSV) expressing either influenza virus hemagglutinin (HA) or neuraminidase (NA) glycoproteins from extra genes inserted in the viral genome. The HA protein was expressed from a site downstream of the VSV glycoprotein (G) gene, while NA protein was expressed from a site upstream of the VSV G gene. The HA protein was expressed at lower levels than the VSV G protein, while the NA protein was expressed at higher levels, as expected from the gradient of VSV transcription that follows the gene order. The HA and NA proteins were transported to the cell surface and were functional as demonstrated by hemadsorption, hemolysis, and NA assays. Biochemical analysis showed that both HA and NA proteins were incorporated into VSV particles at high levels, although there was a preference for incorporation of the VSV G protein over either of the influenza virus proteins. Immunoelectron microscopy of the recombinants showed that the particles derived from the recombinants were mosaics carrying both the VSV G protein and the influenza virus membrane glycoproteins. These results extend earlier studies showing incorporation of the cellular glycoprotein CD4 and two other viral glycoproteins into VSV particles. Our results indicate that there is significant space in the VSV membrane that can accommodate foreign membrane proteins and that the foreign protein can represent as much as 35% of the total protein in the viral envelope. Incorporation of foreign proteins into VSV virions can, in many cases, occur passively in the absence of specific incorporation signals.

Vesicular stomatitis virus (VSV), the prototypic rhabdovirus, has been an important model system for the study of membrane protein and viral membrane assembly. VSV is an especially useful system because the virus can be grown to very high titers in most mammalian cells and is easily purified in large quantities. VSV has a single, negative-strand RNA genome of 11,161 nucleotides containing five genes in the order N-P-M-G-L (3′ → 5′) (see references 33 and 37). These genes are transcribed to form subgenomic mRNAs encoding the five viral structural proteins. The VSV P gene also encodes two additional basic proteins which are found in the cytoplasm of infected cells (35) but do not appear to be important for viral replication in tissue culture (16). The nucleocapsid protein, N, encases the genome to form the RNP core, and the matrix protein probably bridges between the nucleocapsid and the short cytoplasmic tail of the transmembrane glycoprotein, G. The majority of the G protein is exposed on the external surface of the virion and is responsible for receptor binding and membrane fusion to initiate infection. The L and P proteins together form the RNA-dependent RNA polymerase. This polymerase transcribes genomic RNA to make subgenomic mRNAs that encode viral proteins, and it also replicates full-length positive-sense and negative-sense RNAs. As polymerase proceeds along the genome it apparently terminates after polyadenylating each mRNA. After termination, the polymerase reinitiates with only about 70% efficiency, resulting in a transcription gradient (attenuation) that follows the gene order (13).

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We reported recently that VSV can be used as a high-level expression vector (32). This was achieved by introducing an extra transcription unit into a DNA copy of the VSV genome from which infectious VSV can be derived. This vector allows recovery of VSV recombinants expressing an extra gene between the genes encoding the G and L proteins. From our previous studies on the expression of the cellular CD4 glycoprotein or the measles virus glycoproteins from VSV recombinants, we suggested that many foreign glycoproteins might be incorporated at high levels into VSV virions (31). In the research reported here we have extended these results in studies of VSV recombinants expressing the two model glycoproteins of influenza virus.

Previous studies have shown that VSV virions can incorporate foreign glycoproteins after mixed infections with several enveloped viruses, including influenza virus (40), but the extent of the foreign envelope protein incorporation into VSV particles has not been well studied. Influenza A viruses are members of the family *Orthomyxoviridae* and contain segmented negative-strand RNA genomes encoding two different viral envelope glycoproteins. One of these, the hemagglutinin (HA), recognizes receptors containing sialic acid at the cell surface and is also responsible for membrane fusion (12, 20, 38). The other glycoprotein, neuraminidase (NA), removes sialic acid from cells and virions. Removal of sialic acids allows the virus to elute from infected cells and prevents self-aggregation of virions (24). The three-dimensional structures are known for both the trimeric HA and tetrameric NA proteins (36, 39). Because VSV (influenza) pseudotypes can be formed (41), we anticipated that influenza virus NA or HA glycoproteins expressed from VSV recombinants would be incorporated into the VSV envelope. In the experiments described here we sought to determine the extent to which each foreign glyco-

protein could be incorporated and the efficiency of incorporation compared to that for the VSV G protein. We have also used immunoelectron microscopy to examine the distribution of each glycoprotein in the VSV envelope.

VSV recombinants expressing influenza virus proteins could be used as a model for live vaccine development or even as a killed vaccine if the influenza virus glycoproteins were incorporated into the envelope of VSV particles. We chose to study both surface antigens of influenza A virus because these are the primary mediators of protective immune response (7). Antibody to HA is the most important determinant of immunity because it can neutralize the infectivity of influenza virus (4, 6, 10). Although anti-NA antibodies do not neutralize infectivity, they appear to modify the disease and reduce both pulmonary virus and the extent and severity of lung lesions (34).

MATERIALS AND METHODS

Plasmid construction. A plasmid expressing the positive-strand RNA complement of the VSV genome with a site for foreign gene expression (pVSV-XN1) was described previously (32). In this construct foreign genes to be expressed are inserted behind a new promoter between the VSV G and L genes. Here we also employed a second vector, which allows expression of genes between the VSV M and G genes. Construction of this expression plasmid was similar to that described previously for pVSV-XN1 (32) except that the linker for expression of foreign genes was inserted at the unique *Mlu*I site in the 5′ noncoding region of the VSV G gene. The linker regenerated the upstream *Mlu*I site and contained unique *Xma*I and *Avr*II sites followed by a VSV transcription termination signal and start site (see Fig. 1A). Introduction of this linker eliminated the downstream *Mlu*I site. The resulting plasmid was designated pVSV-MXA1, and the presence of the correct sequence was confirmed.

VSV constructs encoding the HA or NA of influenza virus WSN were generated as follows. The HA or NA gene of WSN influenza virus was amplified by PCR from plasmids (15) by using VENT polymerase (New England Biolabs). For HA, the primers used were 5′ACTGCCCGGGCTCGAGAAAA**ATGAAGG CAAAACTACTGG** and 5′AGCTGCGGCCGCGCTAGC**TCAGATGCATATTC TGCAC**. The first primer contains an *Xho*I site (underlined letters) and the first 19 nucleotides of the coding region of the HA gene (boldfaced letters). The second primer contains the last 19 nucleotides of the HA gene (boldfaced letters) followed by an *Nhe*I site (underlined letters). This PCR product was digested with *Xho*I and *Nhe*I and cloned into the *Xho*I/*Nhe*I sites of pVSV-XN1. The resulting plasmid was called pVSVXN1-HA.

For the NA gene, the primers 5′ACGTCTCGAGACGCGTCACT**ATGAATC CAAACCAG** and 5′ACGTGCTAGCCCCGGG**CTACTTGTCAATGGTGAACG** were used. The first primer contains an *Mlu*I site (underlined letters) and the first 15 nucleotides of the NA gene (boldfaced letters). An A (instead of the original T) was introduced in the −3 position relative to the ATG initiator codon to optimize translation initiation (14). The second primer contains the last 20 nucleotides of the NA gene (boldfaced letters) followed by an *Xma*I site (underlined letters). This PCR product was digested with *Mlu*I and *Xma*I and cloned into the *Mlu*I/*Xma*I sites of pVSV-MXA1. The plasmid was designated pVSVMXA1-NA.

Transfection and recovery of recombinant VSV. Transfection and recovery of recombinant viruses were performed essentially as previously described (32). Baby hamster kidney cells (BHK-21, American Type Culture Collection) plated on 10-cm-diameter dishes were infected with vaccinia virus vTF7-3 (9) at a multiplicity of infection (MOI) of 10. After 60 min, plasmids encoding the recombinant VSV-HA or VSV-NA antigenomic RNA and plasmids encoding the N, P, and L proteins were transfected into the cells by using a cationic liposome reagent (29). Plasmid amounts were 10 μg of pVSVXN1-HA or pVSVMXA1-NA, 3 μg of pBS-N, 5 μg of pBS-P, and 2 μg of pBS-L. Subsequent steps were performed as described earlier (32).

Preparation and analysis of protein from recombinant VSV. For metabolic labeling of the VSV and recombinant proteins, BHK cells on a 35-mm-diameter dish (~70% confluent) were infected with wild-type (wt) or recombinant VSV at an MOI of 10 PFU per cell. After 4 h, cells were washed with methionine-free Dulbecco's minimal essential medium (DMEM) and incubated for 1 h at 37°C in 0.5 ml of methionine-free DMEM containing 60 μCi of [^{35}S]methionine. Cell extracts were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (10% acrylamide) and detected on a PhosphorImager (Molecular Dynamics).

For immunoprecipitations, infected BHK cells were lysed in radioimmunoprecipitation assay buffer and immunoprecipitated with rabbit anti-influenza virus WSN serum by using methodology described previously (28). For metabolic labeling of recombinant and wt VSV virions, a monolayer of BHK cells (~70% confluent) on a 35-mm-diameter dish was infected for 3 h (MOI = 10). Cells were washed with methionine-free medium and incubated overnight at 37°C in 1 ml of DMEM and 1% fetal bovine serum lacking unlabeled methionine and containing 100 μCi of [^{35}S]methionine. Cell debris and nuclei were removed by centrifugation at 1,250 × *g* for 5 min, and virus was pelleted from the medium through 10% sucrose at 35,000 rpm in a Beckman SW41 rotor for 1 h. Virus pellets were resuspended in 80 μl of 10 mM Tris-HCL, pH 7.4, and a 10-μl solution was analyzed by SDS-PAGE.

Electron microscopy of VSV particles labeled with gold-conjugated antibodies. BHK cells on 100-mm-diameter dishes (~70% confluent) were infected with VSV (MOI = 0.1). At 20 h postinfection cell debris was pelleted from cultures at 1,500 × *g* for 10 min, and virus was then pelleted and purified by being centrifuged twice through a 10% sucrose solution at 200,000 × *g* for 60 min each time. The virus was imaged essentially as described previously (31). Briefly, virus samples were adsorbed onto carbon-coated grids for 5 min and then blocked with 1% bovine serum albumin (BSA) in phosphate-buffered saline (PBS) for 30 min at room temperature. The grids were then placed on a 50-μl drop of anti-VSV G monoclonal antibodies I1 and I14 (18), anti-HA monoclonal antibodies 431/6 and 523/6 (generously provided by Robert Webster, St. Jude Children's Research Hospital, Memphis, Tenn.), or the anti-NA monoclonal antibody 10C9 (generously provided by Peter Palese, Mount Sinai Hospital, New York, N.Y.). Antibodies were diluted 1:100 in PBS containing 1% BSA. After 1 h, excess antibody was removed by placing grids sequentially onto five 50-μl drops of 1% BSA in PBS and then incubating them with goat anti-mouse immunoglobulin G (Fc) labeled with 15-nm-diameter gold particles (AuroProbe; Amersham). Unbound gold conjugates were removed by five sequential 2-min washes with PBS. The virus-antibody complexes were then negatively stained by incubating the grids for 4 min on 50-μl drops of 2% phosphotungstic acid (pH 7.1). Excess stain was removed, and the grids were air dried. Images of viruses were obtained with a Zeiss EM910 electron microscope.

Hemadsorption assay. Hemadsorption assays were carried out 7 h after infection of BHK cells with VSV or influenza virus WSN which had been propagated as described earlier (5). Monolayers of infected BHK cells were washed twice with PBS, and a 1% suspension of guinea pig erythrocytes in saline was added. After 30 min on ice, the cells were washed five times with PBS and examined microscopically.

For the hemadsorption assay using *Vibrio cholerae* NA (vcNA)-treated cells, infected BHK cells on 60-mm-diameter dishes were washed once with DMEM and incubated with 2 ml of DMEM containing 50 μl of vcNA solution (1 U of NA/ml; Boehringer GmbH, Mannheim, Germany) at 37°C for 1 h as described before (22). The vcNA-treated cell cultures were washed twice with DMEM before the hemadsorption test.

Hemolysis assay. BHK cells grown on 60-mm-diameter dishes (≈70% confluent) were infected with VSV or influenza virus WSN for 5 h. The cells were rinsed with DMEM, scraped into 1 ml of ice-cold PBS, and then sonicated for 15 s. A 200-μl volume of the homogenate was mixed with 200 μl of a 1% suspension of guinea pig erythrocytes in saline, incubated for 15 min on ice, and then briefly centrifuged. A 600-μl volume of 130 mM NaCl–20 mM sodium acetate at different pHs was added to the pellet. The mixture was incubated for 15 min at 37°C and centrifuged, and the optical density of hemoglobin released was measured at 520 nm (30).

NA assay. Five hours postinfection with wt or recombinant VSV or influenza virus WSN, cells obtained from a 60-mm-diameter dish were washed with PBS, resuspended in PBS, and sonicated for 1 s, and the protein concentration was determined by using the Coomassie protein assay reagent (Pierce). NA activities of samples containing the same amount of protein were tested as described previously by using 0.2 μM 4-methylumbelliferyl α-D-*N*-acetylneuraminic acid (Sigma) as a substrate (27). The reaction was stopped after 10, 20, 30, and 40 min, and the fluorescence of released 4-methylumbelliferone was determined in an SLM 8000C spectrofluorometer with excitation at 365 nm and emission at 450 nm.

RESULTS

Expression of influenza virus glycoproteins from two different sites in the VSV genome. We inserted the cDNA sequence encoding the HA protein in the vector pVSV-XN1, which allows recovery of a VSV recombinant in which the HA gene is expressed from a transcription unit between the VSV G and L genes (32). We next inserted the sequence encoding the NA gene into a new plasmid, pVSV-MXA1, which allowed recovery of a recombinant VSV expressing NA from a transcription unit between the VSV M and G genes (Fig. 1). The plasmids obtained were designated pVSVXN1-HA and pVSVMXA1-NA. The gene orders in the VSV recombinants are diagrammed in Fig. 1B.

Our procedure for recovery of recombinant VSV from plasmid DNAs involves simultaneous transfection of the plasmids encoding the full-length antigenomic RNA along with the three plasmids encoding VSV N, P, and L proteins into cells

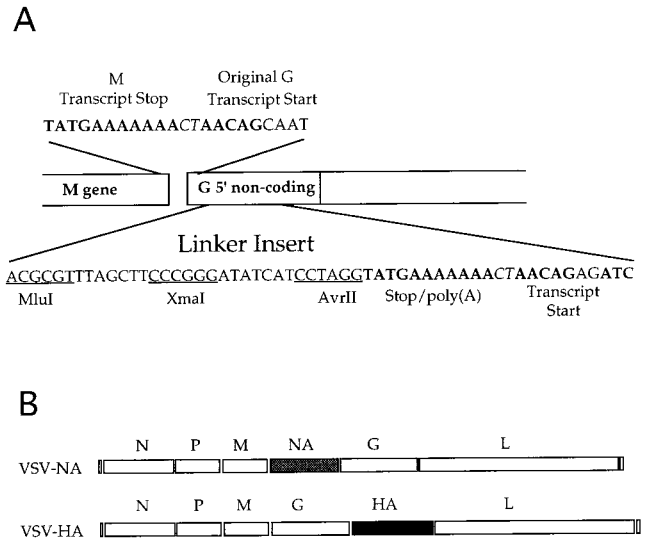

FIG. 1. Vector and virus diagrams. (A) Nucleotide sequence and site of insertion of the synthetic linker encoding transcription stop-start sequences in the pVSV-MXA1 vector. The letters in boldfaced type represent the nucleotides within the consensus sequence that are conserved at all four gene junctions. Intergenic dinucleotides are italicized. The stop/poly(A) sequences as well as the transcription start sequences are indicated. The linker cloned at the unique *Mlu*I site (underlined letters) introduced in the 5′ noncoding region of the VSV G gene and eliminated the downstream *Mlu*I site. (B) Gene orders of VSV-NA and VSV-HA are shown.

infected with vaccinia virus vTF7-3 (9, 17). Recovery of both recombinant VSVs was successful, and expression of influenza virus HA and NA was verified by indirect immunofluorescence of infected cells. Virus stocks for subsequent experiments were then prepared from single plaques.

To determine the protein expression level in cells infected with each recombinant, we infected BHK cells for 4 h and then labeled them with [^{35}S]methionine for 1 h. Because VSV shuts off most host protein synthesis, the viral and additional encoded proteins can be visualized without immunoprecipitation. The results in Fig. 2 show that the VSV L, G, N, P, and M proteins can be seen in cells infected with VSV-HA, VSV-NA (Fig. 2A and B, lanes a), and wt VSV (Fig. 2A and B, lanes c). Furthermore, additional proteins with the sizes expected for HA and NA (Fig. 2A and B, lanes a) were present in the recombinants. The identities of HA and NA were confirmed by immunoprecipitation with a polyclonal anti-influenza virus serum (Fig. 2A and B, lanes b). Also, we noted that VSV G expressed from VSV-NA showed an increase in electrophoretic mobility and formed a sharper band compared to VSV G from wt VSV. This is the result expected if sialic acid were removed from the two N-linked glycans on the VSV G protein by NA.

Quantitation of protein amounts in cell lysates not subjected to immunoprecipitation allowed a comparison of the relative amounts of HA and NA synthesized in cells infected with recombinant virus. The data (correcting for methionine content) indicated that HA was expressed at one-half of the level of VSV G. In contrast, NA expression was 1.8 times that of VSV G protein because of expression from a promoter upstream from the VSV G gene. Because of the polar effects of additional genes upstream of G it was necessary to compare G, NA, and HA expression to expression of the product of an upstream gene (M) in all constructs. The relative expression data quantitated from Fig. 2A and B are given in Fig. 2C. These data show that the presence of the NA gene upstream of G suppresses G expression nearly twofold. It was not possible to obtain accurate quantitation of N and P protein expression because they comigrated in Fig. 2B and because there is a host background protein that comigrates with P. The amount of this host background protein varies depending on the degree of cellular protein synthesis inhibition.

It should also be noted that the amount of attenuation of protein expression is somewhat greater than might be expected based on the 30% of transcription attenuation quantitated at

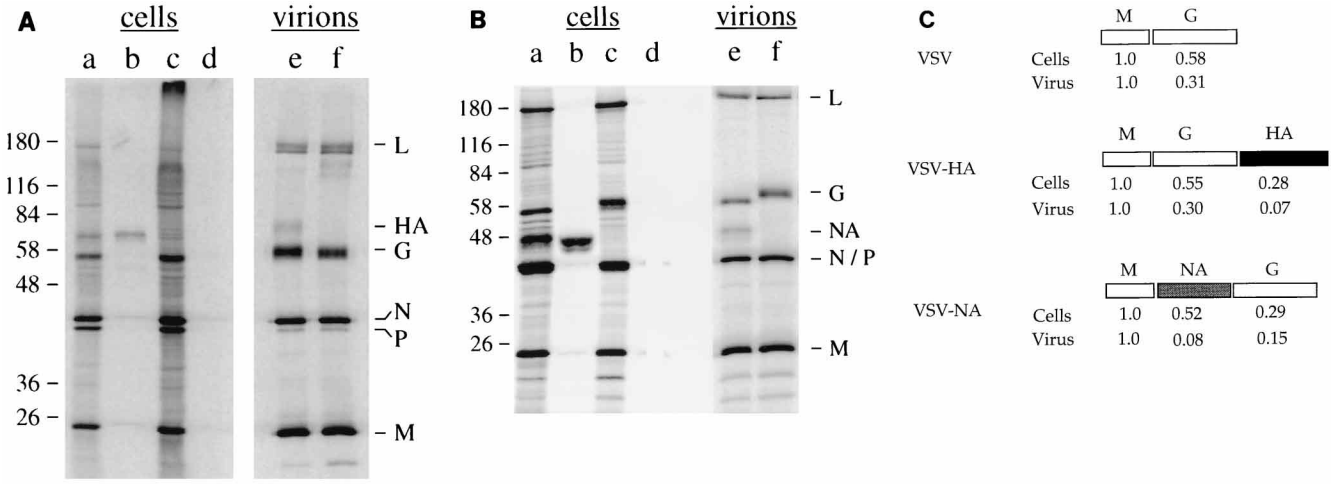

FIG. 2. Expression of influenza virus HA and NA from recombinant VSV and incorporation into VSV virions. Cells infected with VSV-HA and wt VSV were labeled with [^{35}S]methionine. (A) Cell lysates of VSV-HA- and wt VSV-infected cells were analyzed directly by SDS-PAGE (lanes a and c) or after immunoprecipitation with a polyclonal anti-influenza virus serum (lanes b and d). (B) Radiolabeled cell lysates of VSV-NA- and wt VSV-infected cells were analyzed as described for panel A before (lanes a and c) and after immunoprecipitation with a polyclonal anti-influenza virus serum (lanes b and d). Purified virions of VSV-HA and wt VSV (panel A, lanes e and f) and VSV-NA and wt VSV (panel B, lanes e and f), labeled with [^{35}S]methionine, were analyzed as well. Positions of the VSV proteins are indicated. Numbers at left of panels A and B are molecular size markers. (C) Quantitation data obtained from the PhosphorImager of the relative molar amounts of M, G, and HA or NA synthesized in infected cells or incorporated into virions. The amount of M protein was set to 1 and the other values were calculated by correcting for methionine content in each protein.

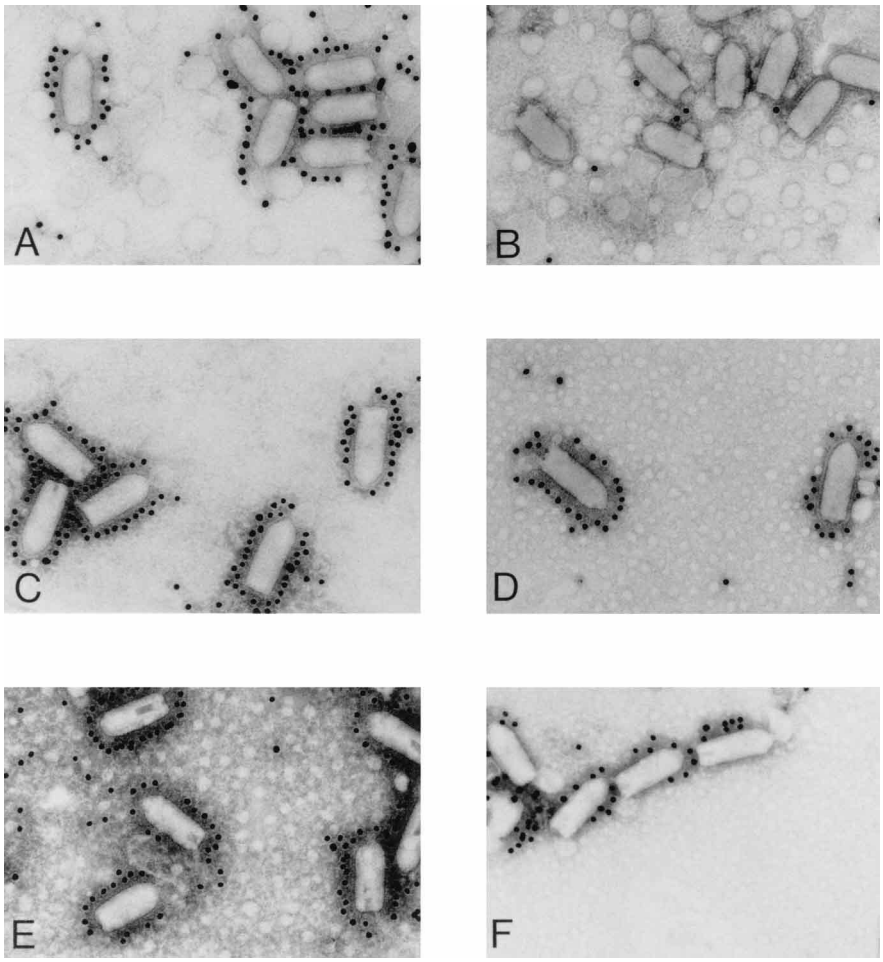

FIG. 3. Electron microscopy of gold-labeled recombinant VSVs. Purified wt VSV (A and B), VSV-HA (C and D), or VSV-NA (E and F) particles were negative stained after being labeled with monoclonal antibodies against VSV G (A, C, and E), HA and NA (B), HA (D), or NA (F) followed by incubation with gold-labeled goat anti-mouse immunoglobulin G. The wt VSV particles shown in panel A are 170 nm long.

each gene junction (13). This discrepancy could result from differences in efficiency of mRNA translation.

Both HA and NA are incorporated at similar levels into VSV particles. Previous studies showed that the human immunodeficiency virus type 1 (HIV-1) envelope protein expressed from a vaccinia virus vector was not incorporated into the VSV envelope unless its cytoplasmic tail was replaced with the VSV G cytoplasmic tail (23), while other proteins do not require the VSV G cytoplasmic tail (31). To determine if influenza virus HA and NA could be incorporated into VSV particles without any specific signals, we labeled cells infected with VSV-HA and VSV-NA with [^{35}S]methionine, purified the labeled virus, and then examined the proteins present by SDS-PAGE. The results in Fig. 2A and B (lanes e) show that both HA and NA were incorporated into VSV particles. Quantitation in Fig. 2C shows that in both wt VSV and VSV-HA, where G protein expression relative to M was the same, G protein was incorporated into virus particles at the same level relative to M protein. The HA protein was expressed at about 50% of the level of G but was incorporated at only about 25% of the G level, indicating preferential incorporation of the G protein. In VSV-NA, where G expression was suppressed twofold compared to that of wt VSV, there was also a twofold drop in incorporation of G into virions. Although the NA gene upstream of G was expressed at twice the level of G, the level of NA incorporation into virions was only about 50% of the level of G protein incorporation, again illustrating the preferential packaging of VSV G into VSV virions. Thus, as for CD4 and measles glycoproteins (31), there was no requirement for the G cytoplasmic tail to obtain these relatively high levels of foreign glycoprotein incorporation into VSV particles.

Distribution of HA and NA in VSV virions. Although biochemical analysis of purified virions showed incorporation of HA and NA into VSV along with VSV G, we wanted to determine what fraction of the particles contained the foreign glycoproteins and if there were changes to the morphology of VSV virions as a result of the inclusion of an additional gene and protein. To address these questions directly, purified wt VSV, VSV-HA, and VSV-NA virions were examined by electron microscopy using gold-coupled antibodies and negative stain (Fig. 3). Figure 3A shows that wt VSV bound anti-G antibodies, while panel B shows that wt VSV did not bind a mixture of three monoclonal antibodies, two directed against HA and one against NA. VSV-HA virions bound anti-G antibodies (panel C) and anti-HA antibodies (panel D). Similarly, VSV-NA bound anti-G antibodies (panel E) and anti-NA antibody (panel F). All particles derived from the recombinants contained VSV G protein in addition to the HA or NA proteins. These results indicate that HA and NA are inserted among the VSV G trimers and that the particles are mosaics

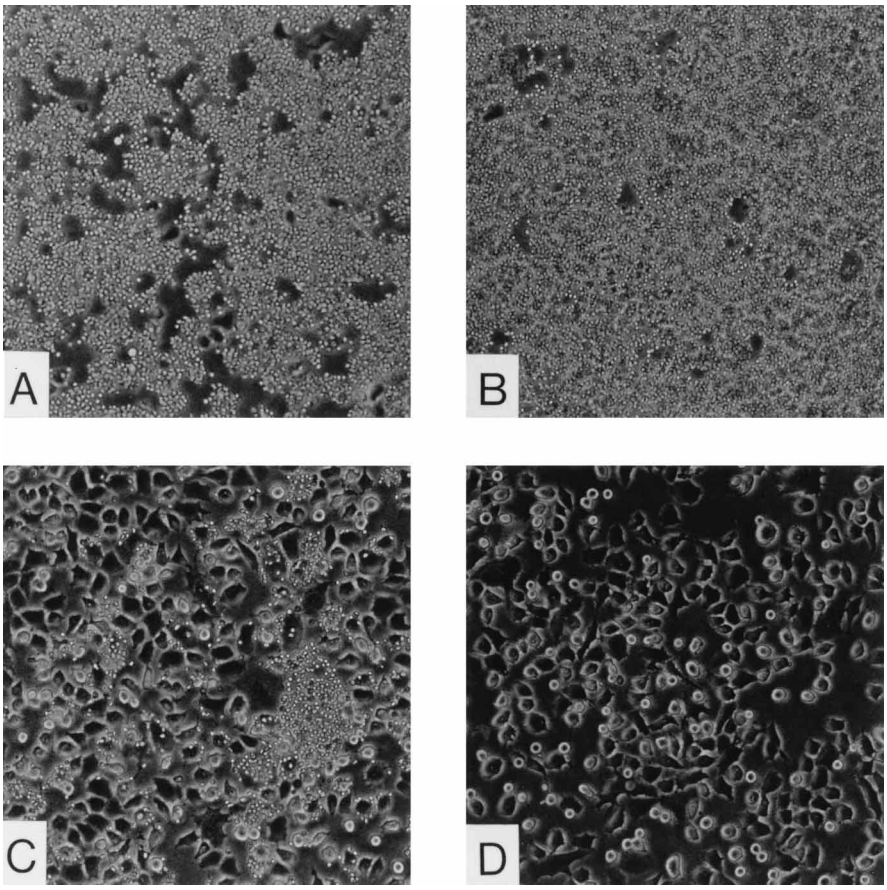

FIG. 4. Hemadsorption of erythrocytes to VSV-HA-infected BHK cells. BHK cells were infected with VSV-HA (A and C), influenza virus WSN (B), or wt VSV (D) and tested for their binding to guinea pig erythrocytes. VSV-HA-infected cells shown in panel A were pretreated with vcNA prior to addition of guinea pig erythrocytes. The larger cells are the infected BHK cells, and the smaller, more refractile cells are the erythrocytes.

containing both proteins. In many particles the influenza virus NA protein appeared to be in patches while HA did not. Similar clustering of NA has also been noted on influenza virus particles (21). The length of the bullet-shaped VSV particles is known to be determined by the length of the helical nucleocapsid containing the RNA. Smaller genomes are packaged into truncated particles (11), and longer genomes have been shown to be packaged into longer particles (31). We measured the images of micrographs of VSV-HA and VSV-NA and found that the VSV-HA particles were about 18% longer than wt VSV particles, whereas VSV-NA particles were about 16% longer. These sizes correspond reasonably well with the additional length of the helical nucleocapsid containing an extra gene of 1,701 nucleotides (HA) or 1,365 nucleotides (NA) within a genome that was initially 11,161 nucleotides.

Functional HA and NA expressed from the recombinants. To determine if the HA protein expressed from VSV-HA was functional, we infected BHK cells with VSV-HA, wt VSV, or influenza virus and examined the ability of infected cells to adsorb guinea pig erythrocytes (Fig. 4). HA from the WSN strain expressed in cells requires treatment with NA or coexpression of NA to show full hemadsorption activity (22). NA removes sialic acid residues from oligosaccharides adjacent to the receptor-binding pocket which interfere with hemadsorption of WSN HA. We therefore treated VSV-HA-infected cells with NA for 1 h at 37°C before performing the hemadsorption assay (Fig. 4A) and obtained much greater hemadsorption compared to untreated VSV-HA-infected cells (Fig. 4C). Cells infected with VSV alone did not bind erythrocytes (Fig. 4D), while cells infected with influenza virus WSN did (Fig. 4B).

Membrane fusion activity of the influenza virus HA results from a conformational change occurring at low pH (1, 2, 38) and can be monitored by measuring hemolysis of guinea pig erythrocytes caused by fusion of membrane fragments containing HA with the erythrocytes (12, 20). Although VSV G protein also undergoes a low pH conformational change and causes membrane fusion (8, 25), it does not cause hemolysis of guinea pig erythrocytes. To measure hemolysis due to HA expressed from VSV, a sonicated homogenate derived from BHK cells coinfected with recombinant VSV-HA and VSV-NA viruses was incubated with erythrocytes. We included the VSV-NA virus in the infection to ensure removal of the sialic acid from the HA molecule. This lysate induced hemolysis below pH 5.8, whereas lysates from cells infected with VSV-NA and wt VSV did not show any hemolysis (Fig. 5). Sonicated lysates from cells infected with VSV-HA alone also did not show detectable hemolysis, but if we pretreated the HA-expressing cells with NA, HA did cause hemolysis (data not shown). The requirement for NA coexpression or NA treatment presumably reflects the need to remove the sialic acid from near the sialic acid binding pocket of HA.

To assay the function of the influenza virus NA encoded by VSV-NA, we performed the enzymatic assay shown in Fig. 6. NA activity was readily detectable in VSV-NA-infected cells.

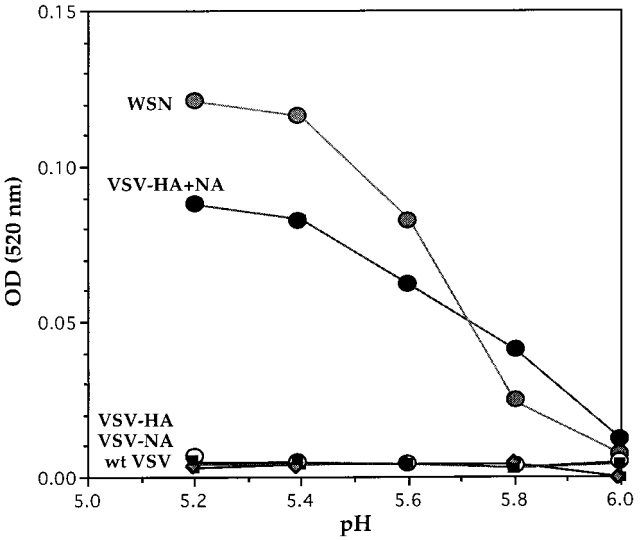

FIG. 5. Hemolytic activity of the HA expressed in BHK cells. Cells were coinfected with VSV-HA and VSV-NA or infected with wt VSV, VSV-HA, and VSV-NA. At 6 h after infection, cell lysates were prepared and sonicated, and a suspension from 2×10^6 cells was assayed for hemolytic activity at the pH values indicated. OD, optical density.

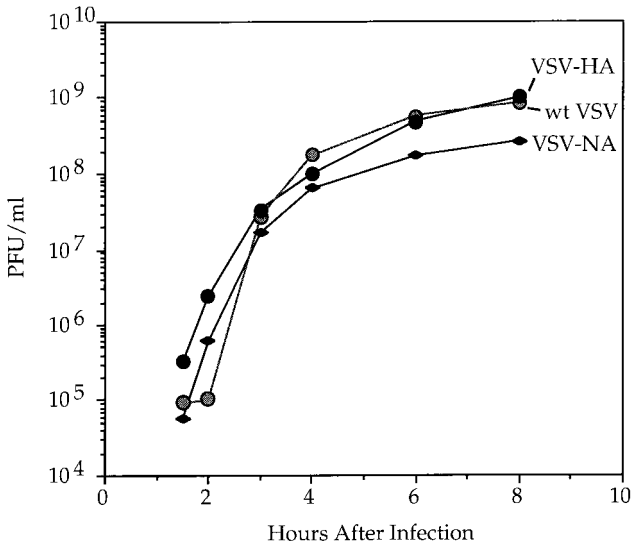

FIG. 7. One step growth curve of recombinant VSV. BHK cells (5×10^6) were infected with wt VSV, VSV-HA, or VSV-NA at an MOI of 10. Supernatant samples were collected at the indicated times postinfection and titered by plaque assays on BHK cells.

When an equal amount of protein from influenza virus-infected cells was assayed, a similar activity of NA was observed. In contrast, VSV-infected cells showed only background levels of NA activity.

Growth of recombinant VSV-HA and VSV-NA. We had observed that the titers of recombinant VSV-NA stocks were typically 5- to 10-fold lower (4×10^8 to 8×10^8 PFU/ml) than wt VSV, while VSV-HA grew to normal titers (4×10^9 PFU/ml). To analyze the virus yield per cell, a one-step growth curve was performed (Fig. 7). BHK cells were infected at a high multiplicity to obtain synchronous infection of all cells. Unadsorbed virus was washed away, and virus released at different times was monitored by plaque assays. Both recombinant viruses replicated at a rate similar to that of wt VSV, but in the case of VSV-NA, the final titer was reduced 5- to 10-fold compared to wt VSV, while VSV HA grew to wt VSV titer. Several independent isolates of the VSV-NA recombinant showed the same phenotype, suggesting that the reduced titer was due to the insertion of the NA gene and not to some secondary mutation picked up in the recovery.

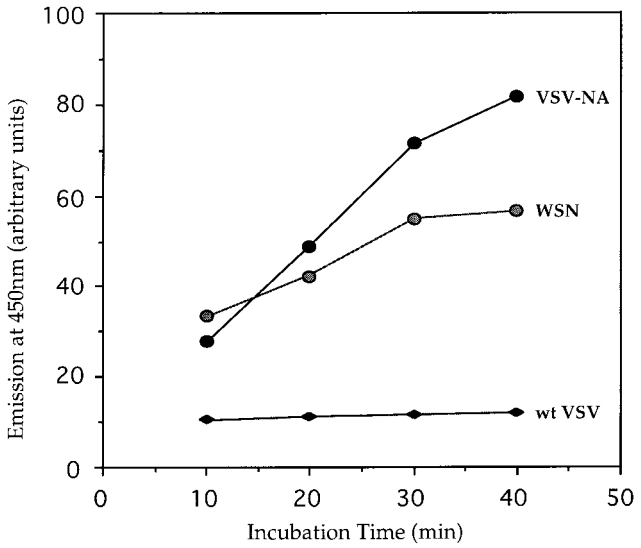

FIG. 6. Activity of NA protein expressed in BHK cells. Time course of NA expression. At 5 h postinfection with VSV-NA, wt VSV, or influenza virus WSN, cell lysates containing the same amount of total protein were tested for NA activity by using 4-methylumbelliferyl α-D-*N*-acetylneuraminic acid as substrate. The amount of released 4-methylumbelliferone and the corresponding NA activity were determined as described in Materials and Methods.

DISCUSSION

We have reported here that high levels of functional HA and NA glycoproteins of influenza virus can be expressed from recombinant VSV viruses. The HA protein was expressed from a promoter downstream of VSV G which was used previously for expression of other genes (31, 32), and the HA was expressed at a level approximately half that of VSV G. A level of NA protein expression nearly twice that of VSV G was achieved in a new vector construct which placed the NA transcription unit upstream of the VSV G transcription unit. These differences in expression levels result from polarity of VSV transcription which attenuates expression of downstream genes (13). Detailed studies of foreign gene expression from multiple sites in the VSV genome are in progress (1a) and have confirmed that different levels of expression obtained for the same gene in different positions result from transcriptional attenuation.

The results presented here extend earlier studies and illustrate that VSV particles can incorporate high levels of additional foreign membrane proteins into the viral envelope. For VSV-HA, the level of HA expression was half that of VSV G in cells, and the level of incorporation into VSV particles was about one-quarter that of VSV G. Thus there was a significant preference for incorporation of VSV G compared to HA, assuming that HA was transported to the cell surface as well as VSV G. In addition we noted that incorporation of HA into VSV did not reduce the level of VSV G relative to nucleocap-

sid protein or matrix proteins, indicating that there is extra space in the envelope for incorporation of the foreign protein.

Cells infected with VSV-NA expressed NA protein at levels nearly twofold higher than VSV G. This twofold decreased expression of VSV G resulted in a twofold decrease in the level of its incorporation into particles. The ability of VSV particles to assemble with less than a full complement of G protein was shown earlier in studies of particles budding early in infection where the G level was reduced threefold relative to that of N and M proteins (19). In contrast, the content of N and M proteins per particle remained constant at different times in infection. Although the VSV G protein expression level was half that of NA in cells infected with VSV-NA, VSV G protein was still incorporated into VSV particles at twice the level of NA, again indicating a preference for assembly of VSV G compared to the foreign protein.

The maximum level of incorporation of foreign membrane proteins (CD4 or measles H proteins) observed in earlier studies was 25 to 30% of the wt VSV G level. In the studies reported here we were able to obtain particles containing influenza virus NA at about 50% the level of VSV G, but this does not represent a real increase in level per particle since G protein expression and incorporation were reduced in this recombinant. These results are consistent with a model in which there is a significant amount of space in the VSV envelope that can be filled by foreign proteins. The high level of incorporation of the foreign proteins appears to depend only on their presence at high levels on the cell surface at the time of VSV budding. High-level incorporation can then occur passively without specific incorporation signals. The HIV-1 envelope is clearly an exception as it is not incorporated into VSV particles unless its cytoplasmic domain is replaced with the cytoplasmic domain of VSV G (13a, 23). In this case there may be signals in the HIV-1 Env protein that cause it to localize in membrane domains separate from VSV budding sites.

Both VSV-HA and VSV-NA virus grew to high titers but the maximal VSV-NA titer was always about 10-fold lower than that of VSV-HA and wt VSV grown in parallel. We do not know why the VSV-NA titers were reduced, but two possible explanations were considered. First, inclusion of the NA gene upstream suppressed VSV G synthesis and resulted in twofold less VSV G per virion. This could result in reduced budding or infectivity of budded particles. Second, cleavage of sialic acid from the N-linked glycans on VSV G might reduce infectivity. However, we favor the first explanation because VSV does not lose infectivity after NA treatment (3). Furthermore, VSV grows to high titers on cell lines unable to generate carbohydrate side chains containing sialic acid (26).

Since one of the future goals of our work is to evaluate recombinant VSVs as potential vaccines, some attributes of this new vector system are worth noting. First, the VSV recombinants express functional membrane proteins that are glycosylated and transported to the cell surface and which therefore can be presumed to be correctly folded and able to present the appropriate epitopes to the immune system. This is an important feature because neutralizing antibodies are normally directed to conformational epitopes of envelope proteins. Second, these proteins are often incorporated into the virus particles, which could permit vaccine applications of killed VSV recombinants. Third, VSV recombinants can be propagated to high titers in mammalian cell lines that have been approved for growth of other vaccines. In preliminary studies, high levels of serum-neutralizing serum antibodies against influenza virus were obtained after immunization of mice with VSV-HA, and immunized mice were protected against a lethal challenge with influenza virus strain WSN (16a).

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